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Generation of circular RNAs and *trans*-cleaving catalytic RNAs by rolling transcription of circular DNA oligonucleotides encoding hairpin ribozymes

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ABSTRACT

A simple new strategy for the *in vitro* synthesis of circular RNAs and hairpin ribozymes is described. Circular single-strand DNA oligonucleotides 67–79 nt in length are constructed to encode both hairpin ribozyme sequences and ribozyme-cleavable sequences. *In vitro* transcription of these small circles by *Escherichia coli* RNA polymerase produces long repeating RNAs by a rolling circle mechanism. These repetitive RNAs undergo self-processing, eventually yielding unit length circular and linear RNAs as the chief products. The transcription is efficient despite the absence of promoter sequences, with RNA being produced in up to 400 times the amount of DNA circle used. It is shown that the linear monomeric hairpin ribozymes are active in cleaving RNA targets *in trans*, including one from HIV-1. Several new findings are established: (i) that rolling circle transcription can be extended to the synthesis of catalytic RNAs outside the hammerhead ribozyme motif; (ii) that rolling circle transcription is potentially a very simple and useful strategy for the generation of circular RNAs in preparative amounts; and (iii) that self-processed hairpin ribozymes can be catalytically active *in trans* despite the presence of self-binding domains.

INTRODUCTION

Catalytic RNAs are now widely studied both for fundamental reasons of understanding their structures and catalytic mechanisms as well as for their potential application to therapy and diagnosis of a wide range of disease states (1–10). Such study requires practical methods for the synthesis of catalytic RNAs of various sizes and sequences. There are two principal strategies now in common use for the synthesis of catalytic RNAs. One of these is automated chemical synthesis (11,12), which has the useful advantage of being able to easily incorporate many types of modified residues. This method is not commonly applied to RNAs longer than ~50–60 nt, however, because yields of full-length product can be limiting. The second approach is run-off transcription (13,14), which readily produces specific RNA strands even hundreds of nucleotides long. Some recognized drawbacks of this approach, however, are that the products are often inhomogeneous in length (15–18) and that it can take considerable manipulation of the DNA template to produce a desired new sequence if it is not short.

A second structural class of RNAs which have come under increasing study, and which are often related to catalytic RNAs, are circular RNAs. These are of interest because circular RNAs are often produced by a number of mechanisms in the biological context (19–21) and because circular RNAs can be more stable against degradation than linear ones (22,23), which increases their practical utility. Methods for construction of circular RNAs are limited not only by the above factors, but also must rely on an added step, which is the molecular strategy for converting a linear RNA into circular form. A number of strategies for laboratory construction of circular RNAs have been reported, making use of protein ligases (24–27), of group I intron splicing mechanisms (28,29) and of reverse ribozyme cleavage (30).

We recently described a new method, rolling circle transcription, which allowed the synthesis of self-processed hammerhead motif ribozymes (31). A number of questions were raised by that work, however. For example, since significant sensitivity to sequence/secondary structure was seen in the transcription, could other ribozyme motifs be produced by this method? Could they be produced in either linear or circular form? Would any ribozyme products with new motifs be able to self-process and cleave other RNAs *in trans*? We now show that hairpin ribozymes can, in fact, be produced by the combined rolling circle/self-processing approach, although the products are different than previously seen. The transcription of these circular templates produces RNAs efficiently, and almost exclusively, two RNA products, circular and linear monomer RNAs, are the result. Moreover, the linear RNAs produced are shown to cleave target RNAs *in trans*, despite the possibility of inhibition by self-complementary domains.

MATERIALS AND METHODS

Synthesis of circular DNAs 67,73,79TRSV and 73HIV

The DNA circles were each synthesized starting with two approximately half-length oligodeoxynucleotides, as indicated below, using sequential enzymatic ligations with splint oligo-

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nucleotides to juxtapose reactive ends. The splint oligomers were 30 nt long, complementary to 15 nt on each of the ends to be ligated.

Synthetic oligodeoxynucleotide precursors (half-circles) for cyclization were: 67TRSV, 5′-pACA ACG TGT GTT TCT CTG GTT GAC TTC TCT GC-3′ and 5′-pTTG CAG GAC TGT CAG GAG GTA CCA GGT AAT ATA CC-3′; 73TRSV, 5′-pTTG AAA CAG GAC TGT CAG GAG GTA CCA GGT AAT ATA CC-3′ and 5′-pACA ACG TGT GTT TCT CTG GTT GAC TTC TCT GTT TC-3′; 79TRSV, 5′-pTGG AAC CAG AAA CAG GAC TGT CAT CGA GTA CCA GGT AAT ATA CC-3′ and 5′-pACA ACG TGT GTT TCT CTG GTT GAC TTC TCT GTT TC-3′; 73HIV, 5′-pCGA AAA CTG GAC TAC AGG GAG GTA CCA GGT AAT GTA CC-3′ and 5′-pACA ACG TGT GTT TCT CTG GTC TGC TTC TCA GGA AT-3′.

The linear oligodeoxynucleotides listed above as well as their corresponding 30 nt splints were synthesized on an Applied Biosystems (Foster City, CA) 392 DNA synthesizer using the standard DNA cycle. They were 5′-phosphorylated using a commercially available phosphoramidite reagent (32) and deprotected with ammonium hydroxide. The crude lyophilized DNAs were used in the ligation–cyclization reactions. A sequential two-step enzymatic ligation method using T4 DNA ligase and the corresponding 30 nt DNA splints was used to construct all four circles. First-step ligation conditions were as follows: 50 µM each half-circle, 60 µM splint one, 0.1 U/µl ligase (US Biochemical) in a pH 7.5 (50 mM Tris–HCl) buffer containing 10 mM $MgCl_2$, 10 mM DTT and 100 µM ATP. The reaction was incubated at 4°C for 14 h. Linear cyclization reaction starting material (pre-circle), when needed, was isolated using preparative denaturing PAGE; otherwise the step 1 solutions were carried on to step 2 (cyclization) without isolation or purification. The conditions for cyclization (step 2) are analogous to those for linear ligation with the following modifications: 1 µM linear precursor, 3 µM splint two and 0.033 U/µl freshly added ligase. The circular products were isolated by preparative denaturing PAGE as decribed (31,33). The reactions were carried out on the 100–300 nmol scale and isolated yields of circular DNA products were: 67TRSV, 10.4%; 73TRSV, 1.3%; 79TRSV, 6.9%; 73HIV, 1.5%. The characterization of the circles was carried out as described (31,33) by nicking with S1 nuclease; the product has the mobility of the linear full-length precursor for cyclization.

Transcription reactions

The single-strand (ss)DNA circles and linear precursors were transcribed with either *Escherichia coli* RNA polymerase or T7 RNA polymerase. Conditions for an internally labeled rolling circle transcription reaction were: 1 µM circle or precircle, 3 U *E.coli* RNA polymerase holoenzyme (Boehringer Mannheim) or 25 U T7 RNA polymerase (New England Biolabs), 0.5 mM ATP, GTP and CTP, 60 µM UTP, 0.30 µCi [α-^{32}P]UTP in a pH 8.1 (25 mM Tris–HCl) buffer containing 20 mM NaCl, 12 mM $MgCl_2$, 0.4 mM spermine–HCl, 100 µg/ml acetylated bovine serum albumin, 10 mM DTT and 12.5 U/ml RNase inhibitor (Promega), in a total reaction volume of 15 µl. Reactions were incubated at 37°C for 1.5 h and the reaction was stopped by addition of 1 vol stop solution (30 mM EDTA, 8 M urea), and frozen at –80°C prior to loading on a 10% polyacrylamide denaturing gel. Gel running temperature was 4°C and xylene cyanol marker dye was run to 24.5 cm from the bottom of the wells.

Sequencing of monomer ribozymes

5′-End-labeled linear monomer RNAs from transcription of each of the three circles were cleaved with RNase T1 to confirm product sequence and length. Unlabeled RNAs were prepared from a rolling circle transcription reaction as indicated above with 12 mM $MgCl_2$ and *E.coli* RNA polymerase in a total reaction volume of 15 µl, but with all four rNTPs at 0.5 mM. Reactions were incubated at 37°C for 7 h and the reactions were stopped by addition of 1 vol stop solution and frozen at –80°C. The entire reaction volumes were loaded on a 10% polyacrylamide denaturing gel run at room temperature with analogous radiolabeled reactions as markers. Unlabeled monomer RNA bands were excised, eluted from the gel and ethanol precipitated.

5′-End-labeling with T4 polynucleotide kinase was done following standard procedures. RNase T1 sequencing was performed on each of the 5′-end-labeled monomers from ethanol precipitation redissolved in water (RNA was not quantitated). Equal volumes of RNA solution were taken for each of the following reactions: control, alkaline hydrolysis and cleavage with RNase T1. Alkaline hydrolysis was done at pH 10.0 for 15 min at 90°C and stopped by addition of 1 vol stop solution and quick cooling on dry ice. RNase T1 cleavage conditions were as follows: 5′-end-labeled RNA and 0.064 U/µl RNase T1 in a pH 3.5 (21 mM sodium citrate) buffer containing 6 M urea and 1 mM EDTA were reacted at room temperature for 10 min and stopped by quick cooling on dry ice. All completed reactions were immediately loaded on a warmer than room temperature 10% polyacrylamide denaturing gel. The bromophenol blue marker dye was run to 28 cm from the well.

Cleavage of oligoribonucleotide corresponding to nt 3605–3618 from HIV *pol*

A 14 nt complementary target RNA was designed to be cleaved by the ribozyme RNAs produced from rolling circle transcription of the designed 73HIV circle. The RNA target oligonucleotide (5′-pCUGUAGUCCAGGAA, corresponding to positions 3605–3618 of HIV-1 *pol* in strain HXB2) was synthesized on the Applied Biosystems instrument using the standard RNA cycle. The RNA was 5′-end-labeled and gel purified by 20% polyacrylamide denaturing gel electrophoresis.

Ribozyme RNAs for study were prepared from rolling circle transcription reactions of 73HIV and 73TRSV ssDNA circles at 12 mM $MgCl_2$, using *E.coli* RNA polymerase in a total reaction volume of 15 µl. Reactions were incubated at 37°C for 5 h and the reaction was stopped by addition of 1 vol stop solution. The entire reaction volume was loaded onto a 10% polyacrylamide denaturing gel and run at 4°C. The corresponding linear and circular monomer and linear dimer bands were isolated and ethanol precipitated.

A time course study of cleavage reactions was carried out for each form of the 73HIV ribozyme isolated. Reaction conditions were as follows: target RNA and specific ribozyme in a pH 7.5 (40 mM Tris–HCl) buffer containing 12 mM $MgCl_2$, 2 mM spermine and 12.5 U/ml RNase inhibitor were reacted at 37°C for various times. Control reactions were carried out at 37°C and contained either target RNA and water, target RNA and cleavage buffer or target RNA with either the linear or circular monomer or linear dimer ribozyme RNAs produced from 73TRSV. Alkaline hydrolysis of the target RNA was under the above

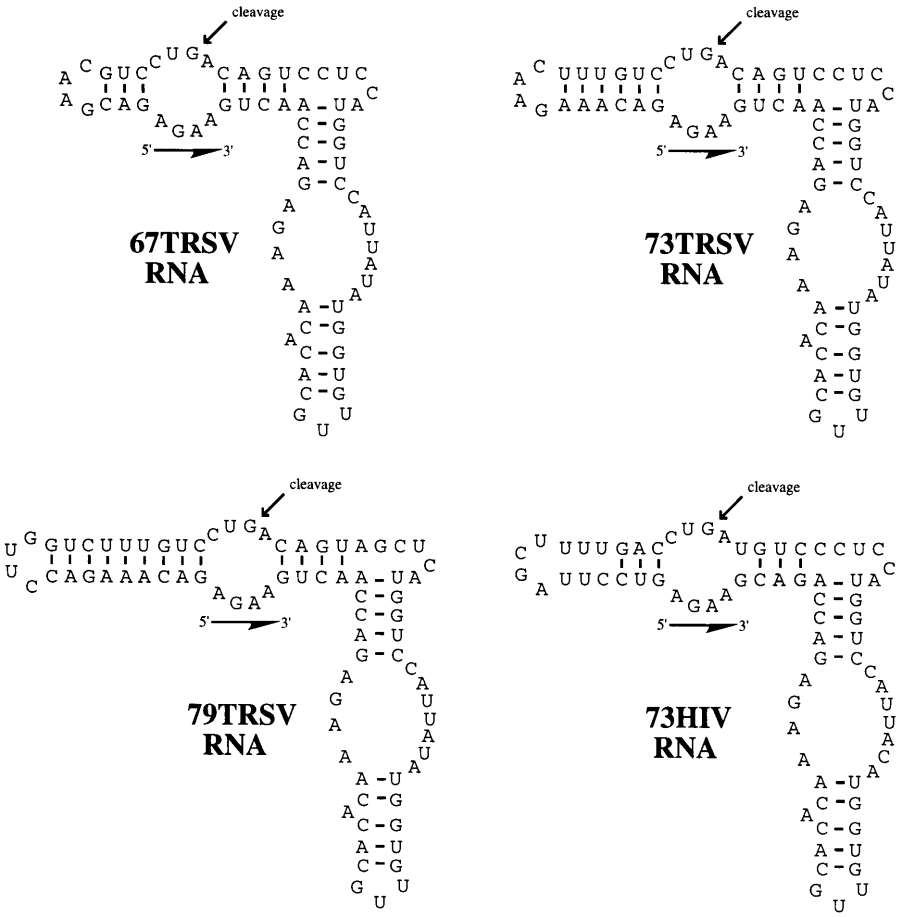

Figure 1. Sequences of monomer hairpin ribozyme RNAs expected after rolling circle transcription of the four circular ssDNAs followed by self-processing. The circular ssDNAs used are the complements of the four RNAs shown. Secondary structures are not known but are shown here in analogy to conserved hairpin ribozyme structure. Helix 1 (referred to in the text) is the 3–9 bp duplex region on the left of each structure.

conditions for 5.5 h at 90°C and was stopped by addition of 1 vol stop solution. All reactions were analyzed on a 20% polyacrylamide denaturing gel with bromophenol blue marker dye run to 15 cm.

RESULTS

Although previous work has shown that certain synthetic circular oligodeoxynucleotides can be transcribed by T7 or *E.coli* RNA polymerases (33,34), there have been a number of cases that are not efficiently transcribed by either enzyme (35), which is apparently due to certain as yet undefined secondary structure preferences for initiation. Therefore, to see whether it is possible to produce hairpin ribozyme motifs by this strategy, we designed a series of four circular ssDNAs ranging in size from 67 to 79 nt (complementary to the RNAs in Fig. 1). To allow for self-processing we incorporated both conserved ribozyme motifs as well as sequences which should be cleavable by them (see scheme in Fig. 2).

The actual hairpin ribozyme sequences encoded in three of these circles (the TRSV series) were derived from the first reported hairpin ribozyme, which was identified in (–) satellite RNA of tobacco ringspot virus, (–)sTRSV (36). To evaluate the required length for maintaining self-processing activity we tested circles of 67 (67TRSV), 73 (73TRSV) and 79 nt (79TRSV) (Fig. 1). The smallest of these is expected to be close to the shortest minimal sequence which would allow for ribozyme activity (37,38). To test the effect of increasing self-complementarity on the product distribution, we incorporated helix 1 domains of 3, 6 and 9 bp respectively. To test whether the binding sequence could be changed to alter the potential cleavage substrate and still retain the ability to be transcribed and self-process, we also constructed a 73mer DNA circle (designated 73HIV, Fig. 1) encoding a hairpin ribozyme targeted to nt 3605–3618 of HIV-1 *pol* (39). Synthesis of these ssDNA circles was carried out in a straightforward manner, producing purified products from crude, unpurified DNA oligonucleotides in yields of 1.3–10.4%.

Transcription was carried out on all four circular DNAs using both *E.coli* and T7 RNA polymerases. We tested both the circular forms and the full-length linear precursor DNAs to evaluate the effect of circularity on the products. Results show (Fig. 3) that all four circles are transcribed well by the bacterial polymerase, but only 67TRSV and 73HIV are transcribed by the phage polymerase. The circular forms of the DNAs are necessary to produce long RNAs; linear DNAs produced only shorter (unit length or slightly longer) RNAs, presumably by non-specific initiation. Transcription of the circles produces RNAs too long to be resolved by the PAGE gel, as well as discrete bands which were later shown to be monomer, dimer and circular monomer products (see below); in addition, slower mobility bands, which are presumably trimer, tetramer, etc., are also visible (Fig. 3). Thus, the results suggest that transcription is followed by self-processing to shorter

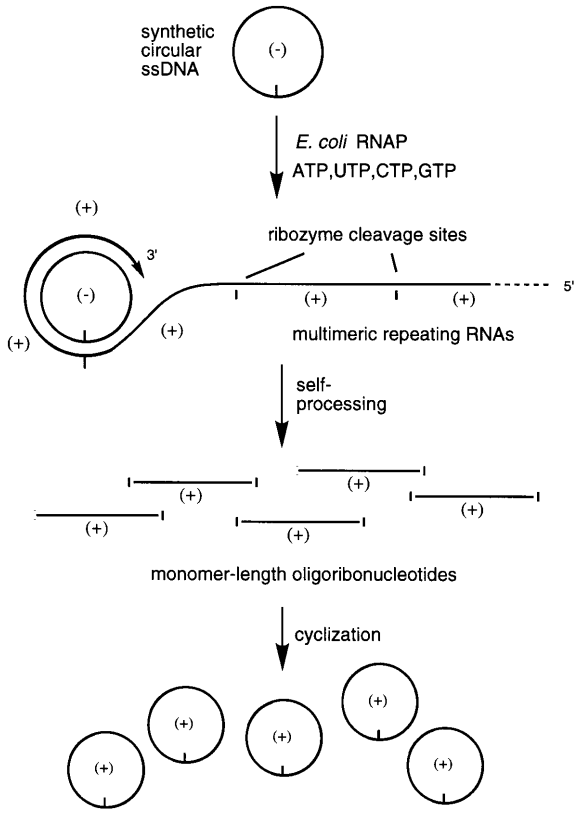

Figure 2. Scheme for rolling circle transcription of circular DNAs encoding hairpin ribozymes followed by self-processing and ligation. Similar steps are also involved in the replication of naturally occurring viroids and virusoids (48).

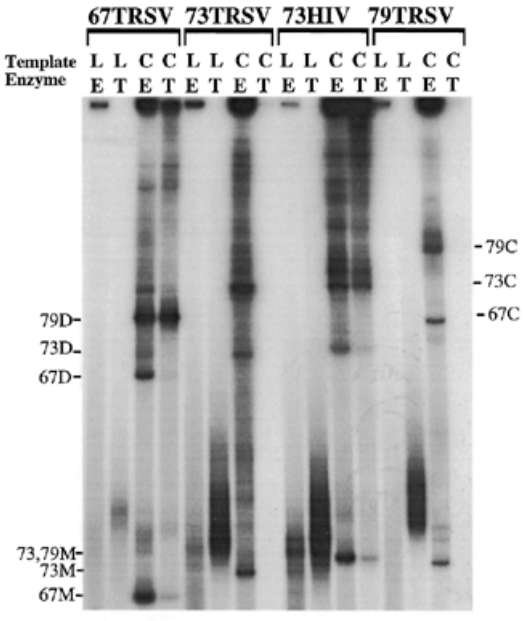

Figure 3. Transcription of linear and circular ssDNAs (see Materials and Methods for sequences) encoding hairpin ribozymes, showing appearance of long RNAs as well as shorter, specific bands. Topology of template DNA is denoted by L (linear) and C (circular); enzyme is denoted by E (*E.coli* RNA polymerase) and T (T7 RNA polymerase). Product RNAs are denoted by length and topology (M, linear monomer; D, linear dimer; C, circular monomer). RNA sequences are given in Figure 1.

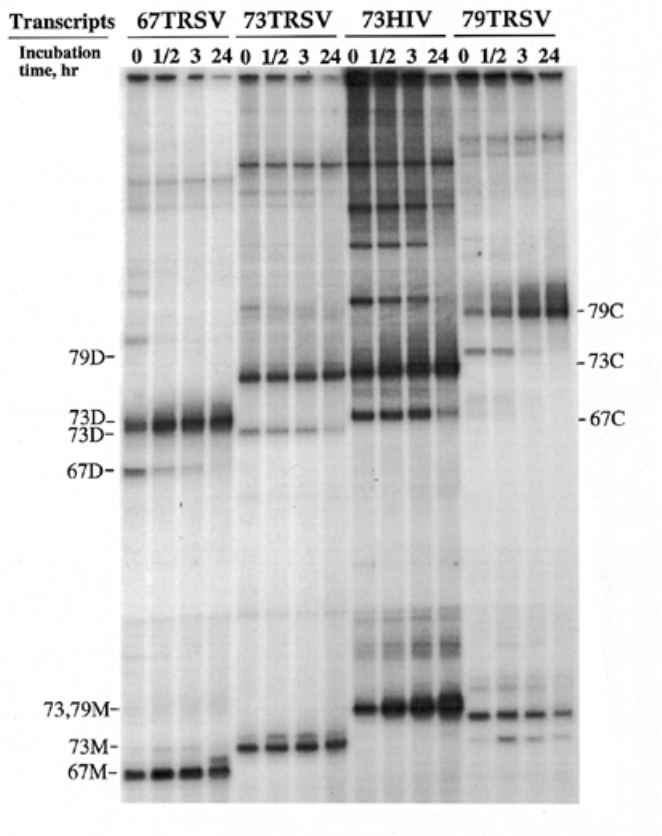

Figure 4. Time course showing self-processing of transcripts, resulting in predominantly circular and linear monomer RNAs. Initial RNA transcripts (Fig. 3) were isolated by ethanol precipitation (without phenol:chloroform extraction) after 1.5 h transcription with the four DNA circles and then were incubated in the presence of 12 mM Mg^{2+} before loading on a denaturing PAGE gel run at 4°C. Product RNAs are denoted by length and topology (M, linear monomer; D, linear dimer; C, circular monomer).

segments and eventually ligation of monomer to circular form (Fig. 2).

Investigation of the efficiency of transcription was done with DNA circle 73TRSV in a non-radiolabeled reaction. RNA products were separated from free nucleotides by size exclusion chromatography using CHROMA-Spin-10 columns and were quantitated by UV absorbance. It was found that after 36 h with 2.5 mM NTPs using 6 U *E.coli* RNA polymerase and 17 pmol DNA circle, the RNA produced contained 7 nmol nucleotide equivalents, which is 400 times the equivalents present in the DNA circle. Thus, each circular template produced polymeric RNAs that are the equivalent of 400 copies of monomer length (73 nt) RNA.

A test of the Mg^{2+} requirements for transcription and self-processing showed that transcription proceeded efficiently with 2 mM Mg^{2+}, and efficient self-processing required 2 mM or greater concentration of the dication (data not shown). To investigate what are the stable products after self-processing, we incubated the product RNAs produced with the four DNA circles after 1.5 h transcription in 12 mM magnesium-containing buffer (Fig. 4). Although in most cases self-processing was largely complete in 30–180 min, there was a small change observable between 3 and 24 h. The final products in all three cases were almost entirely monomer or monomer circle (see below), with slight traces of dimer or dimer circle sometimes visible. The ratios

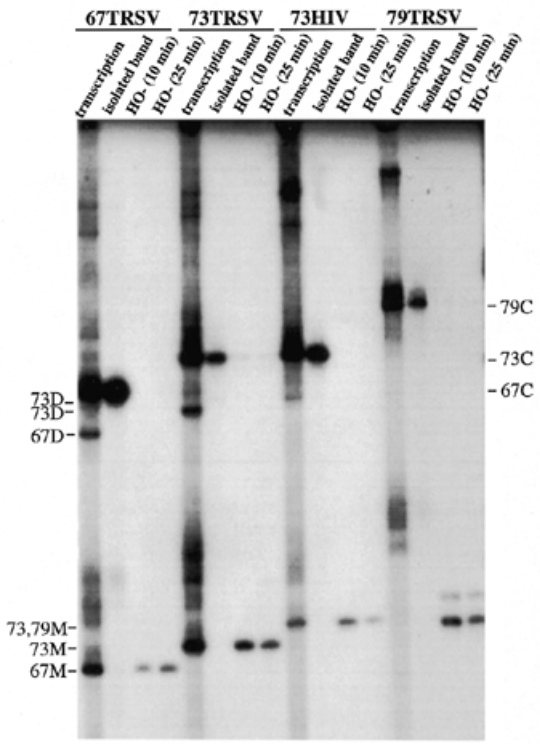

Figure 5. Nicking of suspected circular monomer RNAs to confirm topology. Internally labeled circular monomeric RNAs were excised from a polyacrylamide gel and treated with base (50 mM sodium bicarbonate, 1 mM EDTA, pH 9.0) to induce cleavage. The original transcription mixture is included as a size marker. RNAs are denoted by length and topology (M, linear monomer; D, linear dimer; C, circular monomer).

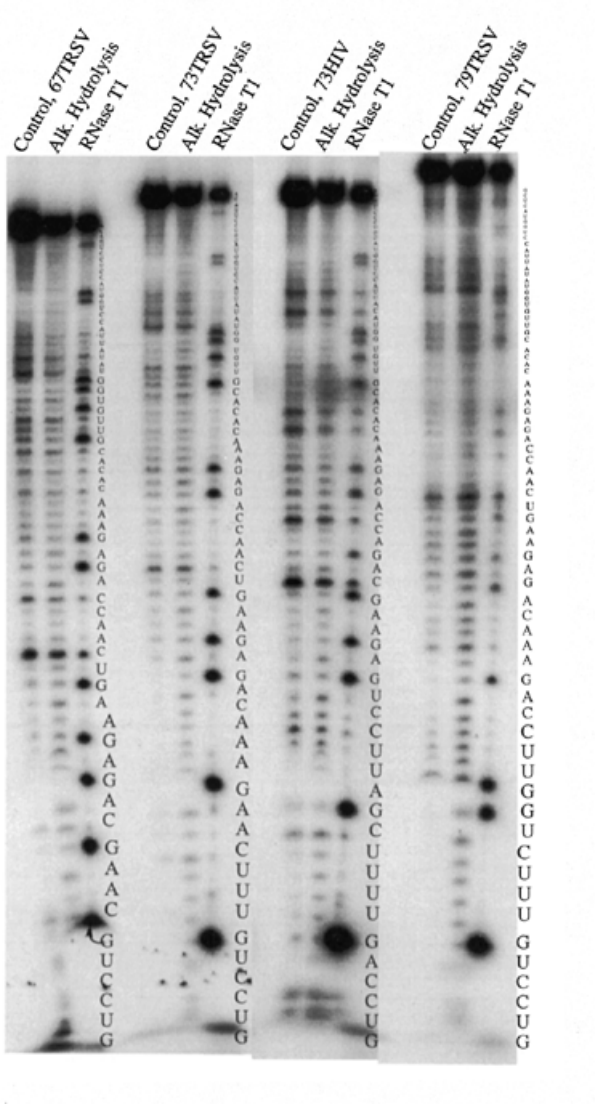

Figure 6. RNase T1 sequencing of monomeric RNAs produced by transcription of the four ssDNA circles in Figure 1. Non-radiolabeled monomer bands were excised from a polyacrylamide gel and subsequently 5′-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and treated either with base (50 mM sodium bicarbonate, 1 mM EDTA, pH 9.0) or with RNase T1.

of circle:linear monomer at 24 h were measured by phosphorimaging and were found to be: 1.25:1.0 (67TRSV); 0.9:1.0 (73TRSV); 0.6:1.0 (73HIV); and 2.5:1.0 (79TRSV).

The putative circular products from transcription of the four DNA circles were shown to be closed (circular) in topology and of monomeric length by nicking under alkaline conditions (Fig. 5); this changed their mobility from that of slow moving circles to single bands having the mobility of linear monomers, which is diagnostic for circular nucleic acids. The putative linear monomer products were characterized by RNase T1 sequencing (Fig. 6). The results confirmed the expected sequence which would result from transcription of these circles followed by self-cleavage at the expected ribozyme target sequences.

The self-processing mechanism which produces the monomer length RNAs results in hairpin ribozyme sequences which contain self-complementary ends that are likely to be at least partially folded to form helices 1 and 2 (the substrate binding domains). This implies possible inhibition of binding of other target RNAs *in trans*. To test whether the major products of transcription and self-processing showed any *trans*-cleaving ability we isolated these three bands (from the 73HIV circle) and incubated them with a synthesized 14 nt RNA which is derived from HIV-1 *pol*. Results show (Fig. 7) that linear monomer and dimer RNAs could in fact cleave this target RNA at the expected site, while the circular monomer could not under these conditions. Similar results were also seen for the RNAs obtained from circle 73TRSV, which cleave an sTRSV RNA target (data not shown) but do not cleave the HIV-1 target (Fig. 7).

DISCUSSION

The results indicate that the rolling circle strategy can be successful in producing amplified amounts of both circular and linear monomeric length hairpin ribozymes, the latter of which can go on to cleave a specific target RNA *in trans*. The data also suggest that it may be possible to influence the product ratio (circular versus linear) in a designed way by altering the length of helix 1 in the hairpin sequence. Short stems are expected to disfavor ligation (yielding primarily linear products in this case), while longer stems would likely favor ligation (yielding primarily circular products) (40). Consistent with this, we observe the greatest circular:linear ratio in the 79TRSV system, which has a 9 bp helix 1. It is not clear, however, why 73TRSV does not give

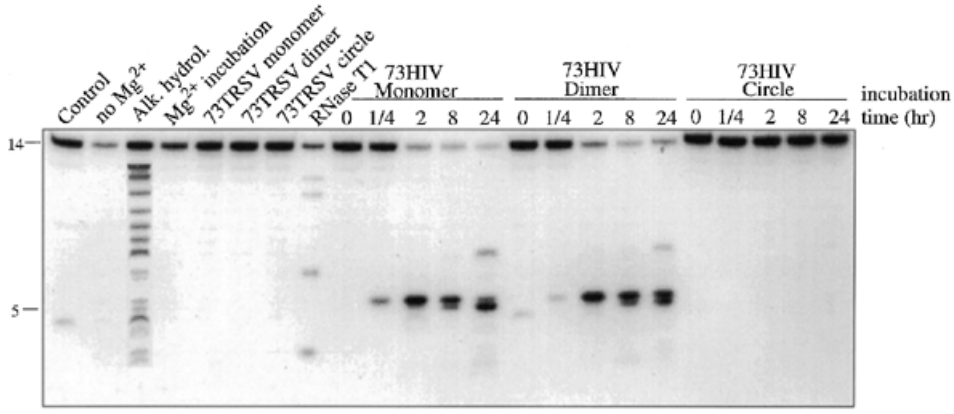

Figure 7. *Trans*-cleavage of a synthetic 14mer RNA target by ribozymes produced from transcription of the designed ssDNA circle. The complementary target for the 73HIV ribozyme is 5′-pCUGUAGUCCAGGAA, corresponding to positions 3605–3618 of HIV-1 *pol* in strain HXB2. Ribozyme RNAs were prepared from 73HIV and 73TRSV ssDNA circles. Expected cleavage site falls after GUA to create a 5 nt labeled product.

an increased circular:linear ratio relative to the 67TRSV case. If it is found to be generally true that long helix 1 stems encourage ligation, then one could in principle insert a given desired sequence into the loop of helix 1 and, after rolling transcription and self-processing, obtain a circular RNA containing this sequence.

The circular products in these reactions may arise from the particular ligating ability of hairpin ribozymes, since recent studies have shown that self-processed hammerhead motif ribozymes generated in a similar fashion are produced almost entirely in linear form (31). A previous study of monomeric self-processed hairpin ribozymes also found that mixtures of linear and circular forms were the result (30). Further studies will be needed in the present system to establish whether ligation to circular form occurs via ribozyme-mediated ligation or whether it is a simple template-directed ligation (41).

The rolling circle/self-processing approach offers an alternative to current methods for producing linear or circular RNAs with well-defined ends. It is well established that linear cassettes of ribozymes flanked by multiple cleavage sites can also be used in *in vitro* transcription to produce self-processed ribozymes with well-defined ends (30,42–47). For example, to produce the 79TRSV RNA one could have designed a linear DNA template ~120–130 nt in length which contains a 20 nt promoter followed by a C-rich initiator sequence, an ~5–10 nt left-hand ribozyme cleavable sequence, then the desired 79mer ribozyme and, finally, a right-hand cleavable sequence. The present approach is different in that it allows the use of much shorter DNA templates (only the actual coding sequence), which is likely to be more straightforward for construction by automated synthesis. In addition, there is no apparent sequence requirement for efficient initiation, as is the case for promoter-driven run-off transcription (13,14).

Hairpin ribozymes have attracted much interest as potential agents for cleavage of disease-related RNAs (1–3,9,10). However, self-processed ribozymes (unlike most small designed hairpin ribozymes) are uniquely structured, in that their substrate binding domains are potentially occupied by their own self-processed ends. *Trans*-cleavage by hairpin ribozymes in this form has not previously been reported, to our knowledge. Our data establish that such a self-processed molecule can in fact cleave a short model RNA sequence derived from HIV-1 *pol*, which suggests that the self-complementary stems unfold rapidly enough to allow binding and cleavage of a target RNA *in trans*. The circular product, less surprisingly, did not show *trans*-cleaving ability. We attribute this to the likelihood that the substrate binding domains of the ribozyme are fully occupied by the intact, covalently closed, self-complementary ends. It may be biologically significant that the monomeric self-processed hairpin ribozymes, such as the linear 73TRSV and 73HIV RNAs, as well as previously described self-processed hammerhead ribozymes (31), can cleave RNA targets *in trans*. This would suggest that naturally occurring viroids and virusoids may have the ability to cleave RNAs from their hosts as well as their own sequences.

The overall process described here with small circular DNA oligomers serves quite successfully to mimic several of the major steps of viroid and virusoid replication (48,49). Those considerably larger natural circular RNAs are apparently transcribed by a plant host RNA polymerase to yield repeating sequences by a rolling circle process, as occurs with our smaller synthetic circles. In both cases, self-processing then occurs, producing monomer length RNAs in amplified amounts. Finally, intramolecular ligation then occurs to yield circular monomeric RNAs as the chief isolated product both in *vitro* (for our molecules) and in plant cells (for natural viroids).

In a practical sense, it seems quite possible that this rolling circle strategy may be useful for the generation of certain biologically relevant RNAs. For example, this may be the shortest available preparative route for small circular RNAs in the ~50–140 nt size range, as well as catalytic RNAs with well-defined ends. This might be particularly helpful in the study of plant pathogens and possibly of biomedically important viroid-like molecules, such as hepatitis delta RNA. The rolling circle approach may also be useful in the preparative synthesis of such RNAs for structural study.

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